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A 32-KDa hydrolase plays an important role in *Paracoccidioides brasiliensis* adherence to host cells and influences pathogenicity

Running title: Role of a hydrolase in *P. brasiliensis* adherence

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ABSTRACT

One of the most crucial events during infection with the dimorphic fungus *Paracoccidioides brasiliensis* is the adhesion to pulmonary epithelial cells, a pivotal step in the establishment of disease. In this study we have evaluated the relevance of a 32-KDa protein, a putative adhesion member of the Haloacid Dehalogenase (HAD)-superfamily of hydrolases, in the virulence of this fungus. Protein sequence analyses have supported the inclusion of PbHad32p as a hydrolase and have revealed a conserved protein only among fungal dimorphic and filamentous pathogens that are closely phylogenetically related. To evaluate its role during the host-pathogen interaction, we have generated mitotically stable *P. brasiliensis PbHAD32*-antisense RNA (aRNA) strains with consistently reduced gene expression. Knockdown of *PbHAD32* did not alter cell vitality or viability, but induced morphological alterations in yeast cells. Moreover, yeast cells with reduced *PbHAD32* expression were significantly affected in their capacity to adhere to epithelial human cells and presented decreased virulence in a mouse model of infection. These data support the hypothesis that PbHad32p binds to extracellular matrix (ECM) proteins and modulates the initial immune response for evasion of host defenses. Our findings point out *PbHAD32* as a novel virulence factor active during the initial interaction with host cells in *P. brasiliensis*.

INTRODUCTION

The adherence of pathogenic microorganisms to host tissues is considered indispensable for their initial colonization and successful infection and dissemination (38). The internalization process has been shown to depend greatly upon the adherence to the host cell surface that generates a cytoplasmatic uptake signal (24). In the case of dimorphic fungal pathogens in which the site of primary infection is generally the lung, the ability to adhere to epithelial cells represents a mechanism by which the infecting agent avoids the entrapment by respiratory tract mucus and, later on, elimination by the action of mucigen ciliary cells (24). *Paracoccidioides brasiliensis* is the etiological agent of Paracoccidioidomycosis (PCM), one of the most common endemic systemic mycosis in Latin American (30, 32). As in other thermodimorphic fungi, *P. brasiliensis* mycelial fragments and microconidia act as the infectious propagules, reaching the lung alveoli where at the temperature of the host's tissues (37^{o}C) it shifts to the parasitic yeast form (6). During this process, adherence of *P. brasiliensis* to pulmonary epithelial cells is considered an essential event.

Extracellular matrix (ECM) proteins have been shown to play an important role during the initial interaction and adherence between host cells and clinically relevant dimorphic fungi, such as *P. brasiliensis*, *Penicillium marneffei, and Histoplasma capsulatum* (17, 18, 22, 25).). In *P. brasiliensis*, the major immunogenic antigen Gp43 (a 43-kDa glycoprotein), detected in the cell wall and as an exocellular compound of both the yeast and mycelial phase, was proven to bind to laminin, a major ECM protein (27, 37, 39). More recently, Gonzalez and co-workers identified a 32-kDa protein in cell wall protein extracts

of both forms of *P. brasiliensis* that was capable of binding to various ECM proteins including laminin, fibronectin, and fibrinogen (14). Additionaly, they demonstrated that this 32-kDa protein is involved in the initial conidial adherence to pulmonary epithelial cells that expressed ECM proteins on the surface, acting as a bridge between both cell types (13).

The main goal of this work was to further characterize this 32-kDa protein and its true role during *P. brasiliensis'* infectious process. Protein sequence analysis revealed a putative adhesion member of the Haloacid Dehalogenase (HAD)-superfamily of hydrolases (PbHad32p). Using antisense RNA (aRNA) technology and *Agrobacterium tumefaciens*-mediated transformation (ATMT) we constructed a mitotically stable *P. brasiliensis PbHAD32*-aRNA strain with consistently reduced gene expression (1, 2). Yeast cells with reduced *PbHAD32* expression were significantly affected in their capacity to adhere to epithelial cells. Moreover, the knockdown strain presented decreased virulence in a mouse model of infection, pointing out *PbHAD32* as a novel virulence factor during the initial interaction with host cells.

Material and Methods

Microorganisms and culture media

P. brasiliensis yeast cells (strain ATCC 60855) were maintained at 36°C by sub-culturing in Brain Heart Infusion media supplemented with 1% glucose (BHI) (Becton Dickinson and Company, Sparks, MD, USA). Unless indicated otherwise, yeast cells were grown in BHI liquid medium at 36°C with aeration on a mechanical shaker and were routinely collected during their exponential phase of growth (72–96 h). Conidia production was carried out as previously described (31).

A. tumefaciens strain LBA1100 (5) was used as recipient for the binary vectors constructed in this study. Bacterial cells were maintained at 28°C in Luria Bertani (LB) medium containing kanamycin (100 mg/ml). *Escherichia coli* XL-1-Blue strain was grown at 37°C in LB medium supplemented with appropriate antibiotics and was used as host for plasmid amplification and cloning (34). Morphological transition from yeast-to-mycelia was performed in BHI liquid medium at 20º (29). The conidia to yeast transition process were carried out by incubating conidia at 36ºC in BHI liquid medium. Samples were collected during the transition process for RNA extraction and quantification of gene expression (11).

To evaluate cell morphology, the strains were exponentially grown, collected, and fixed in a slide and visualized with an AxiosterPlus (Zeiss) microscope. Images were acquired with a Power shot G5 camera (Canon).

Protein sequence analysis

BLAST analysis of the amino acid sequence of the 32 KDa hydrolase protein reported by Gonzalez *et al* (14) was performed at the Broad Institute (*http://www.broadinstitute.org/science/data*). The *P. brasiliensis* genome database was used to obtain the putative complete gene and protein sequence. Multiple sequence analysis of homologous HAD-hydrolases of several organisms was also performed using CLUSTAL 2.0.12 (*http://www.ebi.ac.uk/*): *P. brasiliensis* (Genbank EEH46031.1), *Blastomyces dermatitidis* (NCBI Ref. Seq. XP_002625690), *Histoplasma capsulatum* (Genbank EEH06199.1), *Coccidioides posadasii* (NCBI Ref. Seq: XP_003069355.1), *Penicillium marneffei* (NCBI Ref. Seq. XP_002147878.1), *Aspergillus fumigatus* (NCBI Ref. Seq. XP_753809.1), and *Fusarium graminearum* (NCBI Ref. Seq.XP_385308.1).

Generating *P. brasiliensis* PbHAD32-aRNA strains

P. brasiliensis wild-type (PbWt), strain ATCC 60855, DNA was extracted from yeast cultures during exponential growth using TRIzol (Invitrogen, USA). A Platinum® High Fidelity *Taq* DNA Polymerase (Invitrogen, USA) was employed to amplify aRNA oligonucleotides for *PbHAD32* (AS1, AS2, and AS3).

Plasmid construction for aRNA and ATMT of *P. brasiliensis* were performed as previously described (1, 2). Briefly, the amplified *PbHAD32*-aRNA oligonucleotides were inserted into the pCR35 plasmid under the control of the calcium-binding protein (CBP-1) promoter region from *H. capsulatum* (28). The pUR5750 plasmid was used as a parental binary vector to harbor this aRNA cassette within the transfer DNA (T-DNA). The constructed binary vectors were

introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation as described previously (9) and isolated by kanamycin selection (100 mg/ml). ATMT of *P. brasiliensis* yeast cells was performed using the *A. tumefaciens* cells harboring the desired binary vector as described by Almeida *et al* (2007). A 1:10 yeast:bacteria ratio was employed during the 3-day period of co-cultivation at 28ºC. Selection of *P. brasiliensis* transformants was performed in BHI solid media with hygromycin B (Hyg; 50 mg/ml) during a 15-day incubation period at 36ºC. Randomly selected Hyg resistant transformants were tested for mitotic stability. *P. brasiliensis* yeast cells were also transformed with the empty parental vector pUR5750 as a control during assays carried out in this study.

Gene and protein expression analysis

Total RNA was obtained from PbWt and *P. brasiliensis PbHAD32*-aRNA yeast cells using TRIzol ® (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and tested using a conventional PCR with β-tubulin primers to confirm the absence of chromosomal DNA contamination (12); cDNA was synthesized using 2 µg of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Real-time PCR was done using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The CFX96 Real-Time PCR Detection System (Bio-Rad, Headquarters Hercules, California, USA) was used to measure gene expression levels. *PbHAD32* expression was evaluated in both PbWt and *PbHAD32*-aRNA yeast cells at different time points. Melting curve analysis was performed after

the amplification phase to eliminate the possibility of non-specific amplification or primer-dimer formation. Fold changes in mRNA expression were calculated using the $2^{\Delta\Delta CT}$ formula, where $\Delta\Delta$CT is the difference between the target gene and β-tubulin (house-keeping gene) (21). Each experiment was done in triplicate and the expression level measured in triplicate.

Protein expression analysis was performed by western-blot as described by Gonzalez and co-workers, using a specific monoclonal antibody against PbHad32p (14).

Viability and vitality of *P. brasiliensis* yeast cells

PbWt and *PbHAD32*-aRNA yeast cells were grown in BHI liquid medium at 36°C. After various sub-cultures, we evaluated their viability using ethidium bromide-fluorescence staining (7) and determining colony forming units (CFUs). For this purpose several dilutions of the cultures were plated in BHI supplemented with 0.5% glucose, 4% horse serum, and EDTA 300 mM (19) and CFUs were counted after 7 days of culture.

Vitality was evaluated as the ability to absorbe glucose with later activation of a cell membrane proton pump (35) and subsequent acidification of the media due to released H+. PbWt and *PbHAD32*-aRNA yeast cells were grown in liquid media and measurement of the vitality was made at different time points. Cell samples were collected, washed twice with sterile water (pH 7.0), and suspended in a final volume of 8 ml of water (pH 7.0). Two ml of this suspension were add to a beaker with 38 ml of water and when pH became stable (between 5.5–6), 10 ml of 20% glucose were added. The pH of the

experimental media was evaluated each 3 min up to 60 min to evaluate the increase of H+ in the media.

Adherence of *P. brasiliensis* to A549 cells

The human lung epithelial cell line A549, corresponding to type II epithelial cells from an adenocarcinoma cell line, was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). For the assays, we used confluent monolayers obtained by adding 4 x 10^5 cells per well to 24-well tissue culture plates (Nunc, Kamstrup, Denmark), and then incubated for 24h at 36^{o}C with 5% CO_2 prior to evaluating interaction with PbWt and *PbHAD32*-aRNA yeast cells. A549 cell monolayers were washed once with DMEM culture medium and co-cultured with *P. brasiliensis* yeasts at a concentration of 8 x 10^4 yeast cells per well (corresponding to a ratio of 1:5 for *P. brasiliensis*:A549 cells), and incubated for 1 and 3 h at 36^{o}C 5% CO_2. The supernatant of cultures was then removed, the monolayers were lysed, yeasts adherent to epithelial cells were collected, and dilutions of these suspensions were plated on BHI plates supplemented with 0.5% glucose, 4% horse serum and EDTA 300 mM (19). These results were compared with the number of yeast cells added to each well. The percentage of adherence was expressed as the number of CFUs obtained from each experimental well (*P. brasiliensis* yeast cells and A549 cells) divided by the number of CFUs in the controls (*P. brasiliensis* yeast cells alone). The viability of *P. brasiliensis* yeasts was also evaluated after 24h of infection by determining CFUs and ethidium bromide-fluorescence staining procedures as described above.

To determine cytokine expression we used confluent monolayers and RT-PCR procedures as described above. Total RNA was extracted using TRIzol while ubiquitin was used as the housekeeping gene. We evaluated the expression of interleukins (IL) IL-6, IL-10, IL-12p40 and tumor necrosis factor alpha (TNFα) at different time points (15 and 30 min and 1, 3, 6, 12, 24 and 48 h) of A549 cell line infected with PbWt and *PbHAD32*-aRNA strains. Each experiment was done thrice and the expression level was measured in triplicate.

In vivo infection

Isogenic 8-week-old BALB/c male mice, obtained from the breeding colony of the CIB, Medellín, Colombia, were used in all experiments and were kept with food and water *ad libitum* (33) Recommendations given by the Colombian Government (Law 84 of 1983, Rs No. 8430 of 1993) and the regulations of the European Communities and Canadian Council of Animal Care (1998) were fully complied.

Animals were infected intra-tracheally (i.t.) with 2 x 10^6 *P. brasiliensis* yeast cells harvested at the exponential phase of growth in BHI liquid medium (yeast cell viability above 95%). Prior to infection, cells were washed thrice with PBS, passed through a syringe to eliminate cell clumps, and submitted to Neubauer counting procedures (each mother cell was considered as a single cell).

Statistics

Data are reported as average ± standard error of the mean (SEM), and all assays were done at least three times. All statistical analysis, including analysis of variance (ANOVA), was performed using the SPSS 17.0 statistics program. A

p value <0.05 was considered statistically significant. Survival rate from two independent infections in a mouse model (n = 8 mice for each *P. brasiliensis* strain) was analyzed using Kaplan Meyer and long rank test.

RESULTS

The 32-KDa protein is conserved among pathogenic dimorphic fungi

The previously reported amino acid sequence (14) of the 32-KDa protein was blasted against the sequenced genome of *P. brasiliensis* at the Broad Institute. A match was obtained for a 1716 bp genome sequence, with 2 exons and 1 intron (*PbHAD32*), encoding a protein with 244 amino acids and a conserved Haloacid Dehalogenase (HAD)-superfamily hydrolase domain. Sequence analysis revealed between 58% and 84% identity with other dimorphic and filamentous fungal hypothetical HAD-superfamily hydrolases (Figure 1). No homologues were identified in more evolutionarily distant fungal species (e.g., *Cryptococcus neoformans*, *Candida* sp, and *Saccharomyces cerevisiae*), *Drosophila melanogaster* or mammalian cells.

Knockdown of *PbHAD32* expression

Using aRNA technology and ATMT we generated *P. brasiliensis PbHAD32*-aRNA strains to further study the function of this protein. Three different aRNA oligonucleotides were designed in the 1st (AS1) and 2nd (AS2 and AS3) exons of *PbHAD32* and inserted individually into PbWt yeast cells (Figure 2A). For control experiments, PbWt yeast cells were transformed with the empty vector. A decrease in expression of *PbHAD32* ranging from 72 to 80% was obtained when compared to both the PbWt strain and the strain harboring the empty vector (Figure 2B). No major differences in *PbHAD32* expression were detected among the generated *PbHAD32*-aRNA strains. *PbHAD32* expression in *PbHAD32*-aRNA strains was also determined after 15, 45, and 90 days of sub-culturing of yeast cells, confirming knock-down of gene expression and stable

genomic integration of the T-DNA (Figure 2B). As a control, we also produced conidia from a *PbHAD32*-aRNA strain (AS2) to perform conidia-to-yeast (C-Y) transition and confirm decrease in *PbHAD32* expression after the morphological transition (Figure 2C). Furthermore, protein expression analysis by western blot confirmed decrease in *PbHAD32* protein levels in *PbHAD32*-aRNA strains (Figure 2D). Yeast cells from a *PbHAD32*-aRNA strain generated with aRNA oligonucleotide AS2 was selected for analysis during the remaining assays.

PbHAD32 silencing alters yeast cell morphology without affecting cell viability and vitality

Microscopic observations indicated that *PbHAD32*-aRNA yeast cells were more elongated when compared to the wild-type cells and cells harboring the empty vector (Figure 3A); however, no morphological alterations were observed in conidia or the mycelial form (data not shown). The decrease in expression of *PbHAD32* did not alter yeast cell vitality or viability (Figure 3B). Moreover, no significant differences were detected during batch culture growth of yeast cells between *PbHAD32*-aRNA strains and the controls (data not shown). We also studied the viability of *PbHAD32*-aRNA and PbWt yeast cells at different time points during batch culture. No major differences were observed in either viability or *PbHAD32* expression throughout the assay (Figure 3C).

PbHAD32 plays a role in adherence to human epithelial cells without altering cytokine expression

To elucidate *PbHAD32*'s role in adherence of *P. brasiliensis* to host cells, we infected A549 epithelial human lung cells with yeast cells of PbWt and

PbHAD32-aRNA strains. At 1h of interaction, adherence of *PbHAD32*-aRNA yeast cells was significantly decreased to half, numbers that were approximately maintained after 3h (Figure 4A) and 24h (data not shown). *PbHAD32* expression was also evaluated during infection of epithelial cells. Yeast cells of either PbWt or *PbHAD32*-aRNA strains placed alone in culture medium showed no alterations in gene expression (Figure 4B). However, contrary to what was observed in *PbHAD32*-aRNA yeast cells during infection, a continuous increase in *PbHAD32* expression was detected in PbWt cells up until 12h, decreasing later on until 48h after the initial infection (Figure 4C).

We also evaluated cytokine gene expression during infection of A549 epithelial cells. IL6, IL10, IL12p40, and TNFα were measured at different time points after infection. *P. brasiliensis* wild-type and *PbHAD32*-aRNA strains did not induce cytokine gene expression throughout the assay (at least at measurable mRNA levels).

PbHAD32 is an important virulence factor for _P. brasiliensis_ infection

The relevance of *PbHAD32* during host-pathogen interaction was further evaluated using a mouse model of infection. Animals infected with the *P. brasiliensis* control strain (PbWt) started to die at day 95, with an average survival of 99 days. Contrarily, mice infected with the *PbHAD32*-aRNA strain started to die at day 131 with a survival average of 141 days (Figure 5).

DISCUSSION

One of the pivotal events during infection with *P. brasiliensis* is the interaction and adhesion between fungal and host cells followed by adhesion to epithelial pulmonary cells (24). Although some *P. brasiliensis* molecules that may participate in adhesion to host tissues have been identified (e.g., malate synthase, enolase, thriose-phosphate isomerase, an adaptin-like protein, glyceraldehyde-3 phosphate dehydrogenase, among others), the exact role of these proteins remain uncharacterized due to lack of genetic-based evidence (3, 4, 8, 10, 26).

In the present study we have focused on a 32-KDa protein, PbHad32p, previously shown to play an important role in the adherence of *P. brasiliensis* to host cells and in the subsequent immune response in experimental PCM (13). To further characterize the function of this protein during host-pathogen interaction, we generated mitotically stable *P. brasiliensis PbHAD32*-aRNA strains with significantly reduced *PbHAD32* gene and protein expression as proven by RT-PCR and Western blot analysis. Moreover, reproducibility in assays amongst *PbHAD32*-aRNA transformants generated with different aRNA oligonucleotides (AS1, AS2, or AS3) also supported the hypothesis that observed alterations were due to *PbHAD32* silencing and not random gene disruption via genomic insertion by ATMT. The knock-down of this hydrolase gene did not affect the viability or vitality of *P. brasiliensis* and *PbHAD32-aRNA* strains as both showed similar growth rates, suggesting that *PbHAD32* is not directly involved in cellular processes related to glucose metabolism and yeast cell growth in batch cultures. Interestingly, down-regulation of *PbHAD32*

resulted in yeast morphological alterations, but did not influence mycelial nor conidial aspects. Specifically, more elongated buds were observed suggesting a function for this hydrolase in the maintenance of cell shape during growth. However, the specific mechanism(s) by which this down-regulation affected the morphological alterations was not elucidated in this study. Further investigations should be addressed in order to identify the molecular mechanisms that participate in these kinds of alterations.

The results observed in the present study lead us to hypothesize that both morphological alterations in yeast cells and the reduced expression of *PbHAD32*, probably on their surface, were associated with a decreased capacity of *PbHAD32*-aRNA strains to adhere to human lung epithelial cells. In addition, the absence of detectable mRNA levels of cytokines during interaction with both PbWt and *PbHAD32*-aRNA yeast cells suggests that decrease in the adherence capacity is due to reduced expression of *PbHAD32* rather than from cytokine signaling. Although posing as a relatively passive physical barrier to infection, epithelial cells have been proven to contribute with signaling events during the initial immune response against *P. brasiliensis* infection (23). In addition, previous studies have demonstrated that *P. brasiliensis* strains exhibiting enhanced adhesion to host cells *in vitro* are more virulent (16) and that strains with different yeast cell morphologies are associated with distinct virulence profiles (20, 40). Our data shows that reduction in the expression of *PbHAD32* also leads to significantly increased survival in mice challenged with *PbHAD32*-aRNA yeast cells. Moreover hydrolase expression increased significantly during the first 12h of the epithelial cells interaction with PbWt but

not with *PbHAD32*-aRNA yeast cells suggesting that it is specifically elicited by host stimulation. Taking into account previous reports showing that this 32-KDa protein is mainly located at the cell wall of *P. brasiliensis* (14), our data suggest that decrease in protein levels most likely at the cell surface may lead to reduced capacity to bind to ECM proteins and decreased capability to evade host defenses by modulation of the initial immune response (15). Nonetheless, after the initial adherence to epithelial cells and endocytosis, we do not discard the relevance that phagocytosis by macrophages has in the production of pro- and/or anti-inflammatory cytokines and dissemination of the fungus. The alteration in the ability to adhere to epithelial cells and possible lack of modulation of the immune response during infection with *PbHAD32*-aRNA yeast cells may be assisted by increased phagocytic capacity or enhanced fungicidal mechanisms.

In other clinically relevant dimorphic fungi, interaction with ECM proteins of host cells and subsequent adherence to tissues constitute crucial steps in the establishment of the initial focal infection and dissemination to other organs (17, 18, 22, 25). Protein sequence analysis revealed a conserved homology of this HAD-superfamily hydrolase among human fungal pathogens, both dimorphic (*H. capsulatum*, *Coccidioides* sp, *Blastomyces dermatitidis*, and *P. marneffei*) and filamentous (*Aspergillus* sp and *F. graminearum*), but not with other more distantly related fungi (e.g., *Cryptococcus neoformans*, *Candida* sp, or *S. cerevisiae*). In fact, the high identity among the proteins of the analyzed fungal species coincides with recent research pointing out significant evolutionary events during comparative genomic analysis that phylogenetically group these

fungal human pathogens (36). Altogether, these data further support the relevance of this HAD-superfamily hydrolase in the virulence of *P. brasiliensis*, but also opening the door for further studies related to the relevance of this protein in other fungal human pathogens. Future studies can now be directed at the biochemical evaluation of the possible susbtracts of this enzyme and the differential relevance it may have during adaptation of *P. brasiliensis* to different niches during its life cycle, either as an environmental saprophytic mold or a parasitic yeast cell.

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LEGENDS

Figure 1 – Multiple alignment of HAD-superfamily hydrolase protein sequences from diverse human fungal pathogens: *Blastomyces dermatitidis* (Bd), *Histoplasma capsulatum* (Hc) *Coccidioides posadasii* (Cp), *Penicillium marneffei* (Pm), *Aspergillus fumigatus* (Af), and *Fusarium graminearum* (Fg) revealed 84%, 82%, 77%, 67%, 66%, and 58% identity, respectively, when compared with *P. brasiliensis* (Pb).

Figure 2 – Generation of *P. brasiliensis PbHAD32*-aRNA strains. **A.** T-DNA construct for aRNA silencing of *PbHAD32* in *P. brasiliensis* via ATMT. *PbHAD32*-aRNA oligonucleotides AS1 (1-112 bp of *PbHAD32*; Exon 1), AS2 (175-309 bp of *PbHAD32*; Exon 2), and AS3 (376-536 bp of *PbHAD32*; Exon 2) were amplified, individually placed under the control of the calcium-binding protein (*CBP1*) promoter, and later on inserted into the T-DNA of the binary vector pUR5750 for ATMT of *P. brasiliensis*. **B.** Gene expression levels of *PbHAD32* in PbWt, PbWt transformed with the empty vector (PbWt + EV), and *PbHAD32*-aRNA yeast cells after subculturing in for 15, 45, and 90 days (gene expression levels obtained by RT-PCR were normalized with the internal reference TUB2; * $P<0.05$ when compared with PbWt and PbWt + EV). **C.** Gene expression levels of *PbHAD32* in PbWt and *PbHAD32*-aRNA yeast cells after yeast-to-mycelia (Y-M), production of conidia (M-C) and transition into yeast cells (C-Y) (complete process: Y-M-C-Y) (gene expression levels obtained by RT-PCR were normalized with the internal reference TUB2; * $P< 0.05$ when compared with PbWt). **D.** Western blot analysis of total protein extracts from

PbHAD32 in PbWt and *PbHAD32*-aRNA yeast cells (MW, molecular weight; Lane 1, PbWt; Lane 2, *PbHAD32*-aRNA).

Figure 3 – Silencing of *PbHAD32* leads to distinct *P. brasiliensis* yeast cell morphology without affecting cell vitality. **A.** Microscopic evaluation of PbWt yeast cells and yeast cells from two different *PbHAD32*-aRNA strains generated with different aRNA oligonucleotides (*PbHAD32*-aRNA1 – AS1; *PbHAD32*-aRNA2 – AS2) (magnification 40x). **B.** Vitality of PbWt, PbWt + EV, and *PbHAD32*-aRNA yeast cells. **C.** Cell viability (represented as % on top of bars) and gene expression levels of *PbHAD32* in PbWt, PbWt + EV, and *PbHAD32*-aRNA yeast cells during batch culture growth (gene expression levels obtained by RT-PCR were normalized with the internal reference TUB2; **P*< 0.05 when compared with PbWt and PbWt + EV).

Figure 4 – PbHad32p plays an essential role during adherence to epithelial cells. **A.** Adherence of PbWt and *PbHAD32*-aRNA yeast cells to A549 epithelial human lung cells at different post-infection periods (1 and 3h). The percentage of adherence was expressed as the number of CFUs adhered to epithelial cells divided by the number of CFUs from wells without epithelial cells (**P*<0.05 when compared with PbWt); **B.** Gene expression levels of *PbHAD32* during infection of epithelial cells with PbWt yeasts cells (**P*<0.05 when compared with *PbHAD32-aRNA*); **C.** Gene expression levels of *PbHAD32* in PbWt and *PbHAD32*-aRNA yeasts cells growing in the absence of epithelial cells.

Figure 5 – Silencing of *PbHAD32* decreases virulence of *P. brasiliensis* in a murine model of infection. Representative survival curve of an experimental infection carried out in BALB/c mice via i.t. infection with $2x10^6$ PbWt or *PbHAD32*-aRNA yeast cells ($P < 0.0001$).